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ENDOTOXIN QUANTITATION BY MEASUREMENT OF NON-PRECIPITATED LIMUL--ETC(U)
MAY 78 D A DUBOSE, J M BROWN

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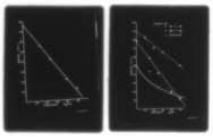

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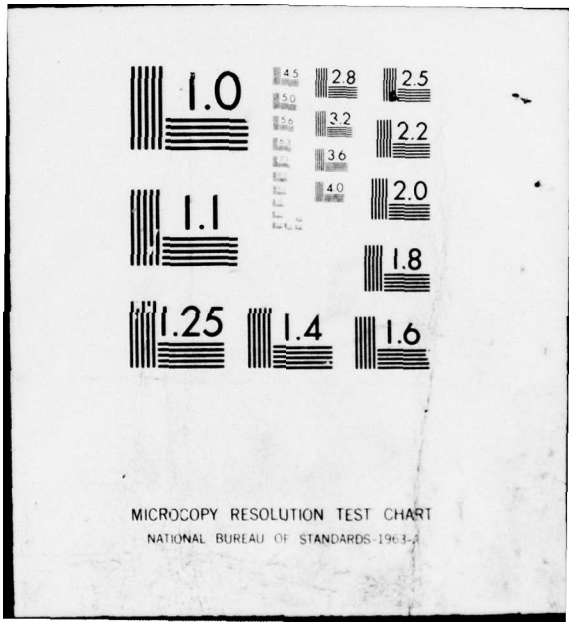
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MICROCOPY RESOLUTION TEST CHART
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REPORT DOCUMENTATION PAGE

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1. REPORT NUMBER: M 27/78	2. GOVT ACCESSION NO.
3. RECIPIENT'S CATALOG NUMBER	
4. TITLE (and Subtitle): ENDOTOXIN QUANTITATION BY MEASUREMENT OF NON-PRECIPITATED LIMULUS PROTEINS	5. TYPE OF REPORT & PERIOD COVERED
6. PERFORMING ORG. REPORT NUMBER	
7. AUTHOR(s): David A. DuBose and Jerry M. Brown	8. CONTRACT OR GRANT NUMBER(s)
9. PERFORMING ORGANIZATION NAME AND ADDRESS: U.S. Army Research Institute of Environmental Medicine, Natick, MA 01760	10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS
11. CONTROLLING OFFICE NAME AND ADDRESS: U.S. Army Medicial Research & Development Command, Washington, DC 20314	12. REPORT DATE: 14 May 1978
	13. NUMBER OF PAGES: 7
14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office): Same	15. SECURITY CLASS. (of this report): Unclassified.
	15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)
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USARIEM-M-27-78

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)
N.A.

DDC RECEIVED JUL 26 1978 F

18. SUPPLEMENTARY NOTES
To be published in: Journal of Pharmaceutical Science

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)
Endotoxin; Limulus Amoebocyte Lysate; Limulus protein; Clottable protein; Polyacrylamide Gradient Gel Electrophoresis; Quantitation by soft laser scanning densitometry.

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)
Using the limulus amoebocyte lysate test a number of procedures have been developed for the quantitation of endotoxin. This report introduces a new method based on the relationship between non-precipitated limulus proteins and endotoxin. Using this relationship standard curves for endotoxin concentration were obtained.

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Endotoxin Quantitation by Measurement of Non-Precipitated Limulus Proteins

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Abstract. Using the Limulus Amoebocyte Lysate (LAL) test a number of procedures have been developed for the quantitation of endotoxin. This report introduces a new method based on the relationship between non-precipitated limulus proteins and endotoxin. Using this relationship standard curves for endotoxin concentration were obtained.

Key phrases. Endotoxin. Limulus Amoebocyte Lysate. Limulus protein. Clottable protein. Polyacrylamide Gradient Gel Electrophoresis. Quantitation by soft laser scanning densitometry.

Since the development of the Limulus Amoebocyte Lysate (LAL) test for the detection of endotoxin (1,2), a number of procedures using LAL have been developed to quantitate endotoxin. These procedures have been based on the development of a solid gel (3), the rate of gel formation (4), or the actual measurement of endotoxin - precipitable limulus protein associated with the gel (5,6). The procedure introduced in this report is based on the relationship between endotoxin concentration and non-precipitated limulus protein left in solution after reaction with endotoxin.

Experimental

The commercial source of lysate employed was Etoxate (Sigma Chemical Co., St. Louis, Missouri, 63178) and 0.1 ml of lysate was reacted with 0.1 ml endotoxin. E. coli (Associates of Cape Cod) endotoxin was used as a standard.

Endotoxin Standard Curves —— A series of endotoxin levels in water ranging from 1.0 ng/ml to 0.0 ng/ml were allowed to react with Etoxate lysate for a period of 15 minutes at 37°C. At this time, the reaction was stopped by agitation of the tubes and lowering the temperature to 4°C. Reaction mixtures were then centrifuged under refrigeration at 25,000 g for 20 minutes. Of the resulting supernatant from each test, 4 µl was applied to Pharmacia polyacrylamide gradient gels PAA 4/30 (PGG) (Piscataway, NJ 08854) and electrophoresed in tris buffer (pH 8.3) at 125V for 15 hours using a Pharmacia GE-4 electrophoresis apparatus and power supply. The PGG were fixed in 30% sulfosalacylic acid and stained by diffusion with solutions of 7% acetic acid containing 0.02% Coomassie blue R

(Polysciences, Warrington, PA 18976). PGG were then destained in 7% glacial acetic acid. The resulting stained limulus protein bands were quantitated using a soft laser scanning densitometer (Biomed Instruments, Chicago, IL 60608). Change in protein level in certain bands was used to develop standard curves for endotoxin concentration.

Estimation of protein molecular weight —— An estimation of the molecular weight (MW) of the proteins in Etoxate was accomplished by measuring migration distance on the PGG. This was related to information, provided with each lot of PGG, about the migration distance of known MW proteins under the electrophoresis conditions stated above.

Results

Figure 1 shows the banding pattern of Etoxate limulus proteins after a 15 minute reaction with three levels of E. coli endotoxin. At least 7 protein bands were found in 3 banding groups on the PGG. One group of proteins appeared to decrease in concentration as endotoxin level increased. The range of MW for these proteins as seen in Table 1 was approximately 40,000 - 50,000. There appeared to be at least 3 protein bands in this low MW group. The 2 high MW groups did not appear to change in concentration.

Figure 2 shows a standard curve for endotoxin concentration established by averaging the level of low MW protein for each endotoxin concentration on three PGG. Low MW protein decreased as endotoxin level increased. R square values

calculated by multiple linear regression showed 97% of the variability in protein level was accounted for by endotoxin level.

Figure 3 shows standard curves for the same E. coli endotoxin using three lots of Etoxate. The standard curve varied with each lot of lysate. Two lots A and B, were consistently found to produce suitable standard curves.

Discussion

These results indicate that there is an inverse relationship between endotoxin activity and the amount of certain non-precipitated Etoxate limulus proteins found in the supernatant which can be used to establish standard curves for endotoxin concentration. After electrophoresis on PGG at least 6 proteins were found divided into three groups. These findings are in concurrence with the report of Young et al. (7). Using a variety of techniques, the MW of clottable protein in limulus lysate has been previously estimated to range between 16,900 and 27,000 (7-9). Electrophoresis on PGG in the present study showed the low MW clottable proteins of Etoxate had an approximate MW ranging from 40,000 - 50,000. Differences in the techniques employed may be responsible for this range of MW for these proteins.

Previous investigators have found a relationship between endotoxin concentration and level of precipitated clottable protein (5,6). Thus, it is not surprising that a similar relationship would be found with non-precipitated clottable protein remaining in solution after a 15 minute reaction of Etoxate with endotoxin.

This method is sensitive and reproducible between 1.0 ng/ml and 0.2 ng/ml of this E. coli endotoxin standard. Within this range of endotoxin, the relationship between endotoxin concentration and level of clottable protein appears to be linear.

In conclusion, the procedures described above can be used to establish standard curves for endotoxin concentration. By comparison to such standard curves unknown levels of endotoxin activity could be determined. In general, it was found that the success of this method was very dependent on the quality of the lysate employed. Some lots of Etoxate were found to be unsuitable for this technique and their lack of adaptability was assumed to be due to a decrease in lysate sensitivity. Perhaps this lack of adaptability of certain lots is related to the seasonal variability in lysate sensitivity previously reported (10). As with other quantitative methods (4-6) not based on the development of a solid gel, this procedure represents an increase in sensitivity over the procedures described by Reinhold and Fine (3).

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Table 1 —— Approximate Molecular Weight of Proteins Found in Etoxate Lysate.

Group #	Protein #	Migration Distance mm	Molecular Weight
	1*	69	40,000
1	2*	68	45,000
	3*	67	50,000
2	4	34	360,000
	5	29	460,000
3	6	28	475,000
	7	27	500,000

*found to decrease in level after reaction with endotoxin

Figure 1 —— Banding pattern of Etoxate lysate after reaction with E. coli endotoxin.

Figure 2 —— Standard curve for E. coli endotoxin using Etoxate lysate. (A.U.=arbitrary units for protein concentration.)

Figure 3 —— Standard curves for E. coli endotoxin with different lots of Etoxate lysate. (A.U.=arbitrary units for protein concentration.)

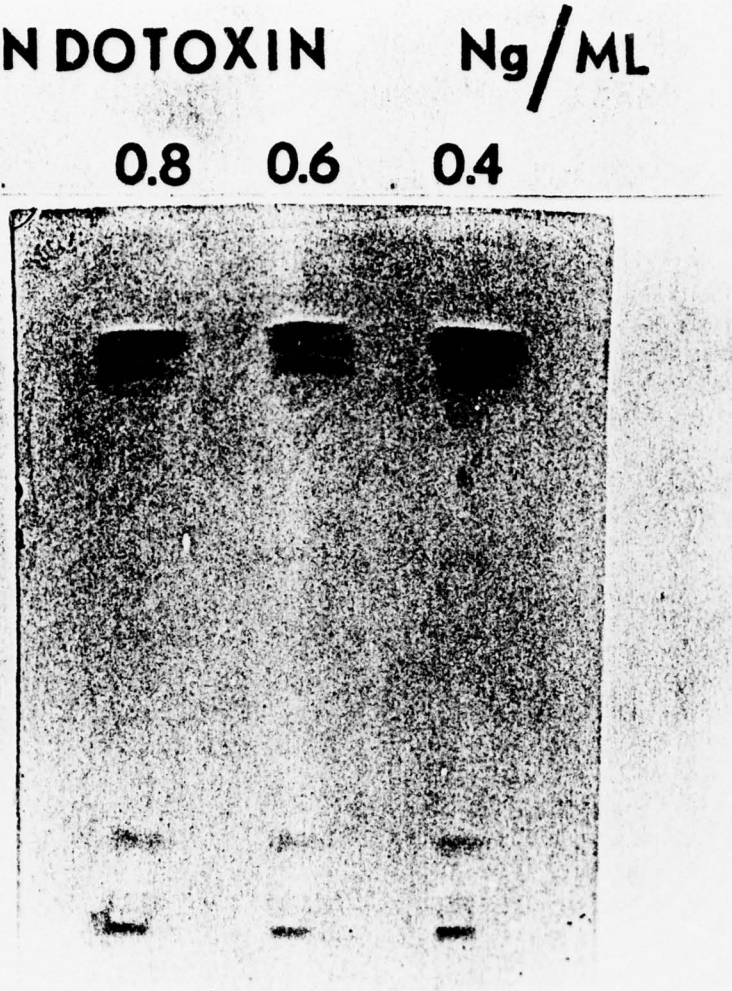

FIGURE #1

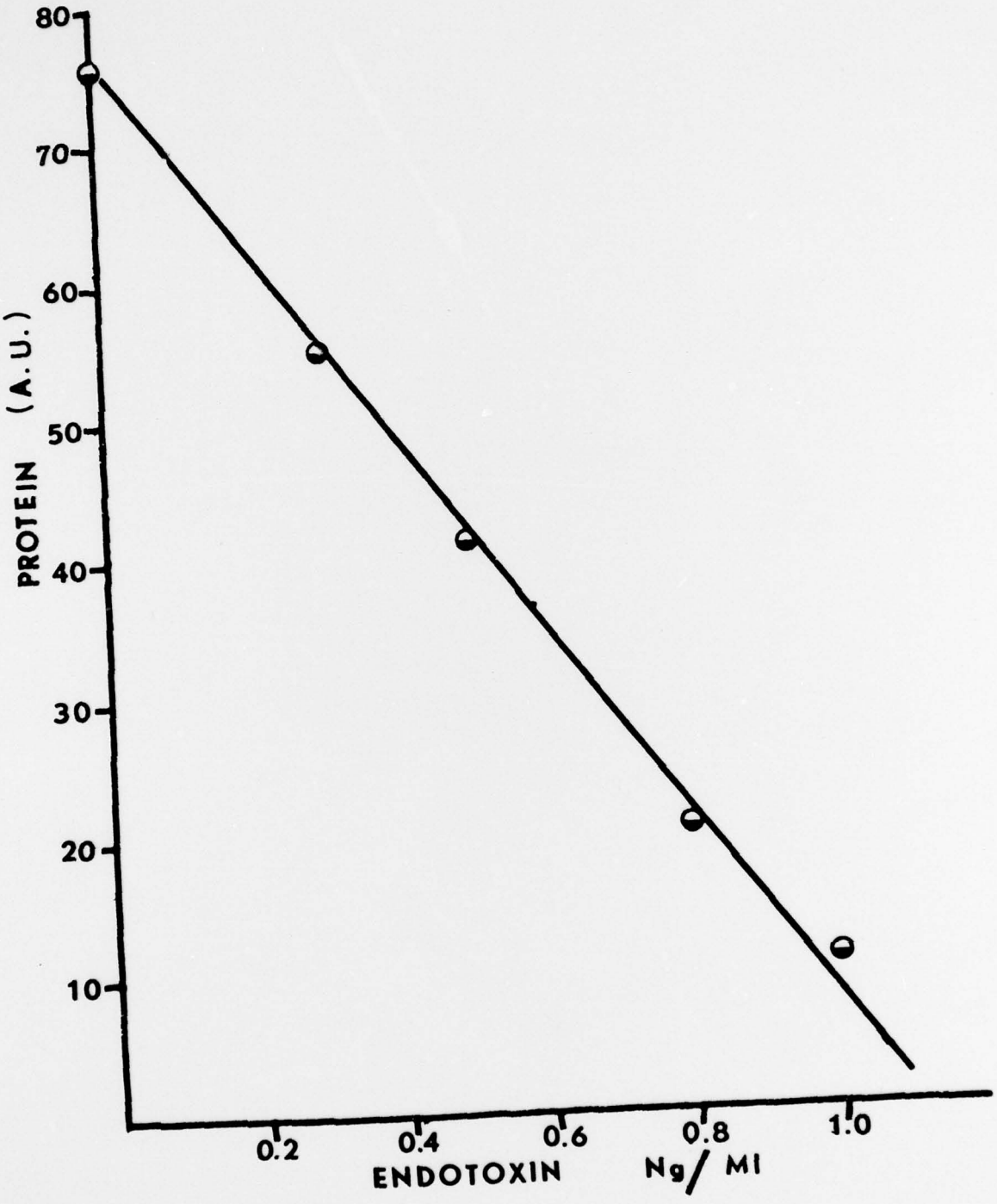

FIGURE #2

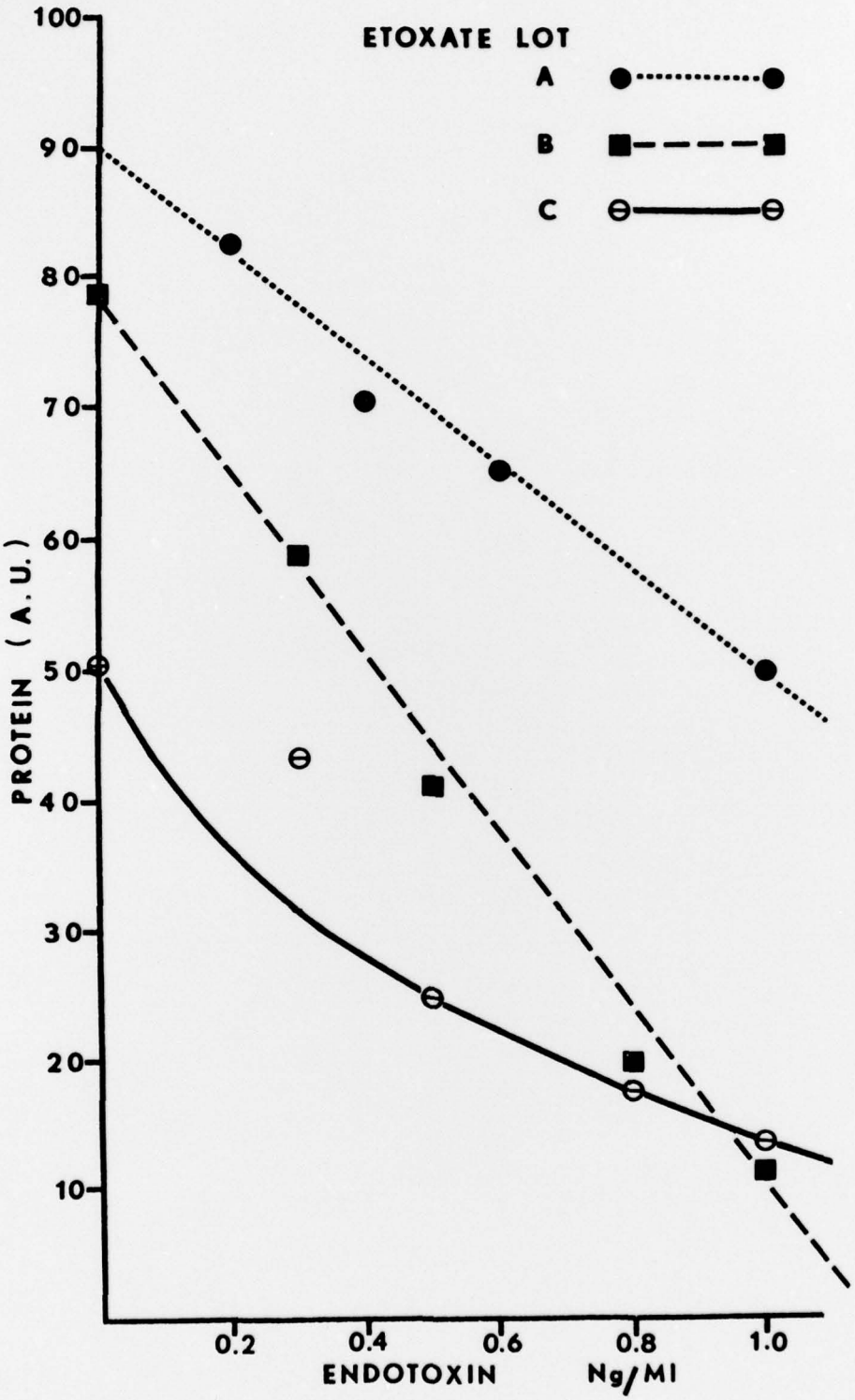
ETOXATE LOT
A
B
C
100
90
80
70
60
50
40
30
20
10
PROTEIN (A.U.)
0.2
0.4
0.6
0.8
1.0
ENDOTOXIN Ng/MI

FIGURE #3